

Physical, chemical and biochemical considerations in the biological degradation of wood

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INTRODUCTION

Microbial degradation of wood should be considered on two levels. From an organismic point of view degradation concerns the interaction of the cell with its microenvironment. At this level, degradation is strongly affected by diffusion of the degradative agents and the uptake of the oligomeric products. From a biochemical perspective, degradation concerns the molecular architecture of the biopolymers, the capacities of the enzymatic catalysts to bind to them and the catalytic turnover rates. Lignin reactivity is limited by the accessibility, heterogeneity, and stability of the polymeric linkages; cellulose reactivity is limited mostly by accessibility and crystallinity; and hemicellulose reactivity is limited by substitution.

The relationships among wood structure, chemistry and biological decomposition have been reviewed many times in articles and occasionally in complete texts (Nicholas, 1973; Loewus and Runeckles, 1977; Rowell, 1984; Higuchi, 1985). If one also considers the specialized texts and monographs on the structure and degradation of individual wood components such as cellulose (Hajny and Reese, 1969) and lignin (Kirk, *et al.*, 1980; Crawford, 1981; Kirk and Cowling, 1984) and volumes from symposia on biomass conversion (*e.g.*, Wilkie, 1975; Gaden *et al.*, 1976; Ghose, 1978; Hollaender, 1981), it is apparent that one cannot add much new detail to what has previously been said. Such, however, is not the purpose. My intention here is to resynthesize past knowledge with a few major recent findings and to relate tissue and polymer structure of wood to mechanisms for its biological and biochemical degradation.

GROSS CHEMICAL COMPOSITION

The chemical composition of wood varies from one species to another. Angiosperms generally contain appreciably more xylan and acetyl, and gymnosperms generally contain significantly more mannan and lignin. Other gross chemical differences exist. Specifically, North American angiosperms average 45% glucan, 18% xylan, 1.2% galactan, 0.6% arabinan, 2.4% mannan, 4.6% uronic anhydride, 4% acetyl, and 22% lignin. North American gymnosperms average 44% glucan, 7% xylan, 2.1% galactan, 1.6% arabinan, 10.4% mannan, 4.2% uronic anhydride, 1.4% acetyl, and 29% lignin (Pettersen, 1984). The structure of the hemicelluloses and lignins differ significantly between the two taxa. The chemical compositions of woods have been extensively reviewed by several authors (Pettersen, 1984; Sjöström, 1981; Fengel and Grosser, 1975; Rydholm, 1965; Browning, 1963). Aside from the presence or absence of extractives, which markedly retard biodegradation, the gross chemical composition of wood actually tells relatively little about its potential for degradation.

DISTRIBUTION OF WALL COMPONENTS

The three principal components of wood are found throughout the wall. Cellulose is organized into laminar crystallites which are bundled into the microfibrils. Each microfibril contains regions of amorphous cellulose interspersed and intertwined with hemicellulose (Fig. 1). The latter is in turn crosslinked to lignin. Most of the cellulose is in the secondary wall layers. Its concentration depends on the relative portions of hemicellulose and lignin.

Distribution of hemicellulose within wood tissue is not homogeneous because vessels and fibers in angiosperms contain appreciably more cellulose while parenchyma cells contain large amounts of glucuronoxylan (Meier, 1985). Presumably the higher hemicellulose content of parenchyma cells assists in distribution of nutrients.

Pentosan content is higher in ray cells than in the total wood (Timell, 1967). In *Pinus sylvestris*, for example, tracheids contain more than twice as much mannose and leu than half as much xylose as ray cells.

Qualitative localization of hemicellulose within the cell walls can be accomplished with selective stains and other techniques, but quantitative distribution is more difficult to establish (Meier, 1985). Hardell and Westermarck (1981) removed individual cell wall layers by microdissection and isolated three fractions from tracheids of spruce: The (M + P) fraction had substantially higher arabinose and galactose content, but xylose and galactose varied little throughout the wall, indicating that the hemicellulose content in cell walls is proportional to the cellulose and not to the lignin.

Lignin is the encrusting material. It is found in the highest concentration in the middle lamella, but because the volume of the middle lamella is small, most of the total lignin is found in the secondary wall (Table 1).

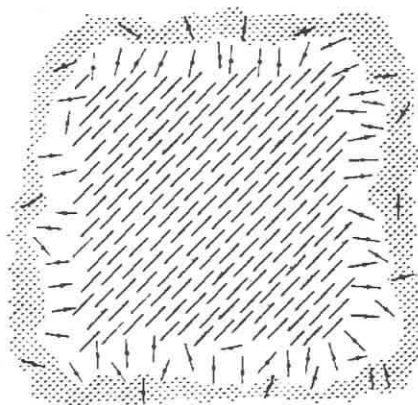


Fig. 1. Cellulose (—), hemicellulose (---) and lignin (· · ·) complex in wall.

Table 1. Distribution of lignin in two gymnosperm species (Saka and Goring, 1985).

Species	Morphological region ^a	Tissue volume ^b		Lignin			
		E	L	% total		Conc (g/g)	
				E	L	E	L
Black spruce	S	87	94	72	82	0.23	0.22
	ML	9	4	16	10	0.50	0.60
	ML _{cc}	4	2	12	8	0.85	1.00
Douglas fir	S	74	90	58	78	0.25	0.23
	ML	10	4	18	10	0.56	0.60
	ML _{cc}	4	2	11	6	0.83	0.90

^aS= secondary wall, ML = middle lamella, ML_{cc}= middle lamella, corner region

^bE= earlywood; L=latewood

The total amount of lignin in the secondary wall is greater in latewood than in earlywood, primarily because of the increase in the thickness of the secondary wall layer. Even though

the concentration of lignin increases in latewood middle lamella, the proportion of lignin found in this region decreases mostly because the middle lamella is much thinner.

COMPONENT CHEMISTRIES

Lignin is a stable, highly cross-linked aromatic macromolecule arising through dehydrogenative free radical polymerization of *p*-coumaryl, coniferyl and sinapyl alcohols. Gymnosperm lignin is made up principally of coniferyl alcohol (guaiacyl lignin) whereas angiosperm lignin is made up from approximately equal amounts of coniferyl and sinapyl alcohols (syringyl/guaiacyl lignin) (Freudenberg and Neish, 1968; Adler, 1977). *p*-Coumaryl alcohol is found principally as a precursor to the lignin of grasses and in reaction wood (Freudenberg and Neish, 1968). Although the lignin polymer can be described by a combination of three monomeric units, the large number of interunit linkages which arise through free radical polymerization leads to a great deal of structural complexity. A few prominent substructures comprise most of the interunit linkages (Fig. 2). By far the most common (40-50%) is the arylglycerol- β -aryl ether link (A). The next most common (\gg 10%) are the phenyl coumaran (B) and biphenyl (C) substructures. The biosynthesis and chemical characterization of lignin has been presented extensively elsewhere (Higuchi, 1985; Fengel and Wegener, 1984; Adler, 1977; Sarkanen and Ludwig, 1971; Freudenberg and Neish, 1968).

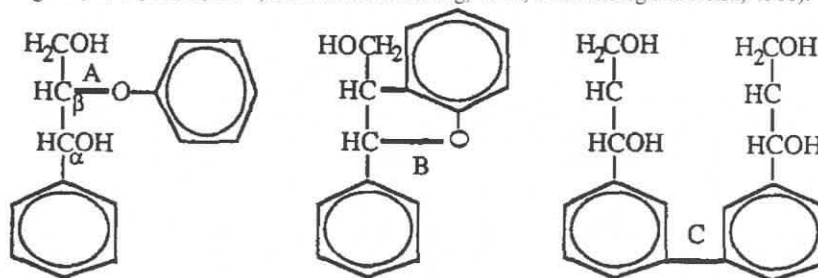


Fig. 2. Major interunit linkages found in lignin (Alder, 1977).

The relative stability of the chemical bonds determines the reactivity of the polymer as a whole. Although different bonds are reactive under different conditions, all of the linkages in lignin are stable in comparison to other biological polymers. Lignin is condensed by strong acid, but degradation occurs in strong alkali or in the presence of strong oxidizing agents. Any linkage to the α carbon will exhibit relative instability. In fact, the rate of alkaline degradation of open-chain *p*-hydroxybenzyl-aryl ethers is too fast to be measured (Miksche, 1980). *p*-Hydroxyphenylcoumaran structures (B) are also relatively easily degraded with cleavage of the $\text{C}_\alpha\text{-O}$ ether linkage and the formation of a double bond between $\text{C}_\alpha\text{-C}_\beta$, but the degradation rates of all the other linkages are much lower than these. The most stable structure is the aromatic ring. Probably because of the greater stability of the $\text{C}_\beta\text{-O}$ and biphenyl linkages, the lignin linkage most readily oxidized during biological degradation is the $\text{C}_\alpha\text{-C}_\beta$.

Lignin is not simply deposited between cell wall polysaccharides, but is chemically linked to the hemicelluloses with a frequency of about 1 per every 35 phenyl propane units (Obst, 1982). Direct linkages to glucan are relatively rare except at the non-reducing terminus. Carbohydrates are common contaminants in lignin preparations, even when highly purified: and degradation of lignocellulose by polysaccharidases leads to the isolation of polymeric lignin with carbohydrate attached (Adler, 1977). Methylation analysis has shown that the predominant link to carbohydrate is through arabinoxylan. Lignin bonding can occur at the C-2 and C-3 of the xylose monomer and at the C-5 of arabinose side chains (Minor, 1983). Arabinose, xylose and 4-O-methylglucuronic acid are the most common sugar moieties to which lignin is cross-linked. Benzyl ester, benzyl ether and phenyl glycosidic linkages are probably involved (Fengel and Wegener, 1984).

Cellulosic microfibrils are the most conspicuous element of the plant cell wall. The β -(1,4)-D-glucose chains which make up the cellulose polymer can be arranged in both parallel and antiparallel crystallites; but based on what we now know about the biosynthetic (Hassid, 1969; Lloyd, 1984) and biodegradative (Chanzy and Henrissat, 1985) mechanisms and x-ray crystallographic studies (Gardner and Blackwell, 1974), the parallel arrangement is almost certainly the form found in nature.

The structure of cellulose is derived from its chemical character and its biochemical origin. Each chain of cellulose is held straight by O6-O2 and O3-O5 intrachain hydrogen bonds and adjacent chains are bonded into a flat sheet by O3-O6 hydrogen bonds. Adjacent laminar sheets are held together by van der Waals' forces (Gardner and Blackwell, 1974; Marchessault and Sundararajan, 1983). The cellulose chains are packed with the plane of the glucose ring oriented diagonally with respect to the flat planes of the microfibril (Caulfield, 1971).

Interspersed throughout the cellulose crystallite are amorphous regions in which crystallinity is very much reduced. These regions could result from the curvature of the cell membrane during cellulose synthesis. A crystallite forming as the cellulose synthetase complex moves across the surface of the cell membrane would eventually jut out away from the membrane too far for the complex to maintain order in it. An amorphous region would be formed between the crystallite and the complex, and a new crystallite would be organized out of the packing of the synthetase complex.

The resistance of the cellulose crystallite to hydrolysis is attributable to the rigidity of the rings held tightly by the crystal structure. The rotational energy barrier encountered in ring flexure is a fundamental factor in controlling the rate of hydrolysis (Harris, 1975), and the principle extends to conformation and substitution of the glycosides involved. If, for example, one compares the relative rates of hydrolysis for C1- β -methyl pyranosides and β -(1,4)-linked disaccharides, they fall in the same order as determined by the ring stability of the glycone (the non-reducing terminal glycoside which is released to form a reducing group) and not the aglycone (the reducing terminus) (Table 2).

The glycosidic linkages of a hemicellulosic polymer of glucose is more stable than that of mannose, and a polymer of mannose is more stable than one of xylose. The steric hindrance attributable to the crystallinity of the cellulose polymer combined with the greater stability of the bond, accounts for why hemicellulose is so much more readily hydrolyzed than cellulose. This picture is complicated somewhat, by the fact that glucuronosides - substituents in angiosperm xylans - are hydrolyzed at about one thirtieth the rate of glucosides (Harris, 1975).

Table 2. Relative hydrolysis rates of methyl β -pyranosides and β -(1,4)-linked disaccharides (Harris, 1975).

Methyl pyranoside of	Relative rate	Disaccharide	Relative rate
β -D-glucose	1.0	Cellobiose	1.5
β -D-Mannose	3.0	Mannobiose	2.7
β -D-Galactose	4.8	Lactose	3.6
β -D-Xylose	5.8	Xylobiose	11.0

The major hemicellulose of angiosperms is *O*-acetyl-4-*O*-methylglucuronoxylan. A second hemicellulose found in much lower proportions is glucomannan. In gymnosperms, the major hemicellulose is *O*-acetylgalactoglucomannans. Gymnosperms also contain arabino-4-*O*-methylglucuronoxylan which is similar to the polymer found in angiosperms (Timel, 1967; Whistler and Richards, 1970). The main chain of angiosperm xylan is composed of 6-membered pyranose rings in the chair conformation linked β -(1,4) as in the case of cellulose. Because xylose does not possess the C6 hydroxyl, O2-O6 intrachain and O6-O3 interchain hydrogen bonding is not present. In xylan, O3-O5' hydrogen bonding form the chain into a twisted ribbon with a repeat unit of xylotriose (Marchessault and Liang, 1962). Straight-

chain, unsubstituted xylan is relatively insoluble. In wood cell walls, xylan possesses monomeric side branches consisting of D-glucuronic or 4-O-methylglucuronic acids linked a glycosidically to the O2 position with an average frequency of 1 per 10 xylose residues (Timell, 1967). Also, native xylan is frequently acetylated on the O3 or the O2 position with a frequency of about 7 in 10 xylose residues. This acetylation makes the polymer much more soluble in water (Biely, 1985; Whistler and Richards, 1970). The significance of such acetylation to enzymatic degradation is frequently overlooked because the acetyl groups are commonly removed during isolation. The main chain of the xylan polymer contains one β -(1,3)-linked branch point about every 75 residues (Whistler and Richards, 1970).

Glucmannans are the major hemicellulose of gymnosperms where they comprise about 15-17% of the total dry weight. In Contrast, they are minor components of angiosperms, generally comprising only 3-5%. In angiosperms, they contain glucose and mannose most commonly in a 1:2 ratio linked in a β -(1,4) straight chain (Timell, 1967). In gymnosperms, they occur in close association with cellulose (Whistler and Richards, 1970). The majority of glucmannans contain a few galactose residues as non-reducing end groups. The ratio of galactose, glucose, and mannose in gymnosperms is commonly 2:10:30 (Timell, 1961). Arabinogalactans are found principally in larch wood, but they are also minor occasional components in angiosperms. The polymer consists of a β -(1,3)-galactose main chain with β -(1,6)-galactose and (1,6)-arabinose (furanose) side branches, and it is highly water soluble (Whistler and Richards, 1970). Although larch arabinogalactan is a minor and atypical hemicellulosic component of wood, it is frequently employed in xylanase assays, presumably because of its easy isolation and solubility. For the taxonomic distributions and structures of hemicellulosic polymers see specific treatments (McNeil, *et al.*, 1984; Stephen, 1983; Rees, 1977; Towle and Whistler, 1973; Timell, 1967; Whistler and Richards, 1970).

MICROSTRUCTURE AND POROSITY

Porosity and microstructure are critical in determining accessibility of enzymes to their polymeric substrates. The relationship between enzyme diffusion and substrate accessibility has been shown in a number of ways. For example, reducing particle size greatly increases surface area and speeds up the enzymatic hydrolysis of cellulose (Millet *et al.*, 1975). Grinding or cutting wood to open all the lumina to attack will expose considerably more surface than is available in whole wood. The total surface area reaches about $1 \text{ m}^2 \text{ g}^{-1}$. Once the lumina are accessible, however, relatively little further increase in surface area occurs following further grinding, because the cell wall thickness is very small compared to its length (Stone *et al.*, 1969). Particle size is not the only factor important in enzymatic depolymerization. Enzymatic accessibility depends on the structure of the wall itself.

The pore volume of cell walls can be estimated from the water content. In the dry state, the wood cell wall is non-porous (Stone *et al.*, 1966). Green wood, however, is wet, so it follows that in its wet state, the wall is swollen above its dry volume by an amount equal to the volume of water it contains. At the fiber saturation point, *i.e.*, when the walls are completely saturated with water but before any free water is found in the cell lumens, the volume of water contained in the fiber is equal to the total pore volume. In the case of sprucewood, the total pore volume is about 0.48 ml g^{-1} , virtually all comprised of pores less than 50 \AA and 60% less than 10 \AA in diameter (Stone and Scallan, 1968). The molecular pore distribution of individual wall components of native wood is poorly characterized. However, it is known that the cellulose crystallite excludes water by hydrogen bonding. The water content of lignin is also probably low by virtue of its aromatic structure. Hemicellulose, on the other hand, has a high affinity for water; so it seems likely that the bulk of the pore structure in wood cell walls will be found in this fraction.

A distinction should be made between the effects of substrate crystallinity and those of microporosity. Susceptibility of pure cellulose to enzymatic hydrolysis is inversely related to its degree of crystallinity (Sasaki *et al.*, 1979; Caulfield and Moore, 1974; Fan *et al.*, 1980). However, in any practical situation, enzymatic hydrolysis is not carried out with pure

cellulose; so considerations other than crystallinity prevail. Recent studies by Grethlein (1985) using wood which had been partially hydrolyzed by dilute acid have shown a linear correlation between pore volume accessible to a protein with a nominal diameter of 51 Å and the initial rate of cellulose hydrolysis obtained with *Trichoderma reesei* cellulase. The crystallinity index of the substrate was not related to the rate of hydrolysis. Likewise, in an examination of enzymatic hydrolysis of a series of treated lignocellulosic substrates, the rate of cellulose hydrolysis did not correlate with crystallinity (Puri, 1984). Also, in a study of the initial hydrolysis rates obtained with *Clostridium* and *Trichoderma* cellulases acting on six different cellulosic substrates, Weimer and Weston (1985) concluded that the rate limiting factor for both cellulases was accessibility and diffusion rather than crystallinity.

The relationship between component microstructure and enzymatic degradation has been studied most extensively with cellulose. Stone *et al.* (1969) employed solute exclusion to measure the pore volume in cotton linters swollen to varying degrees in phosphoric acid. The swollen cellulose gels were hydrolyzed with *Trichoderma* cellulase having a reported molecular weight of 60 kd. They found a linear relationship between the initial reaction rate and the surface area within the gel which was accessible to a sphere of 33-40 Å dia., corresponding nominally to a protein of 40 to 70 kd (Stone and Scallan, 1968; Cowling and Brown, 1969; Weimer and Weston, 1985). However, neither the pores nor the enzymes are spherical.

Stone *et al.* (1969) visualized the pores in swollen cellulose not as circular in cross section, but as slit-like spaces between the cellulose lamellae. The elongated morphologies of cellulases have been recognized for some years, with the earliest work dating to that of Whitaker *et al.* (1954). Of 27 cellulases summarized by Cowling and Brown (1969), the ratio of length to width averaged 5.9 ± 0.33 and the apparent molecular weights ranged over 10 fold. One can therefore conclude that elongate shapes for cellulases are more characteristic than their sizes.

Even very small enzymes can possess relatively large binding sites. For example, lysozyme (molecular weight = 12.5 kd) is known from x-ray crystallographic studies to bind six pyranose rings (Imoto *et al.*, 1972), and indirect evidence has been given to show that a β -(1,3)-D-glucanase with a molecular weight of 27 kd has a binding site encompassing at least 14 β -(1,3)-D-glucose rings (Jeffries and Macmillan, 1981). Contrary to what one might expect, however, the groove for the binding site of lysozyme runs across the short axis of the enzyme. If endoglucanases follow a similar pattern, the portions of the enzyme sticking out on the sides adjacent to the binding site could be involved in separating laminar sheets of the cellulose crystallite.

DEGRADATION OF WHOLE WOOD

Microbial degradation of whole wood occurs principally by fungi. Although bacteria apparently possess much of the biochemical machinery necessary for biodegradation of the wood components, they do not form mycelia, and hence, cannot propagate through the tissue structure or translocate nutrients into the wood or from one region to another as decay progresses.

The various components found in different types of cells and within cell walls affect the degradation rates. Electron microscopic studies of wood degraded by white-rot fungi have shown that in some instances, parenchyma cells can be almost completely degraded, leaving vessels behind (Otjen and Blanchette, 1984). The different rates of degradation could be related to the different hemicellulose compositions of the two cell types. The relationship between wood structure and microbial degradation is somewhat ambiguous because of the many separate variables such as lignin content and composition, the presence of extractives, the density of the tissue structure, moisture content, and the relation of these variables to the invading organism (Wilcox, 1973). The most resistant wood species generally have high extractives contents and have been summarized (Scheffer, 1973), and Wilcox (1973) has summarized the organisms characteristic of wood decay.

Wood degradation is limited by diffusion. Diffusion of oxygen and CO₂ through the wood is restricted, and the interior of a decaying tree is essentially anoxic (Jensen, 1969). Heartrot

fungi appear to possess special metabolic capabilities which enable them to grow under the restrictive conditions found in the center of a log (Highley and Kirk, 1979). Diffusion of gases occurs principally through the cell lumens. If the cells are filled with water, essentially no oxygen transport occurs. In fact, methanogenic bacteria - a taxonomic group intolerant of even traces of oxygen - have been found in the heart of decaying, water-saturated cottonwood, (Zeikus and Ward, 1974). Diffusion of gas out of the wood is limited and concentrations of CO₂ can be as high as 20% of the total gas volume in wood which is undergoing fungal decay. (Hintikka and Korhonen, 1970; Jensen, 1969). As decay progresses, porosity increases and oxygen availability increases as well.

The intracellular lumen and wall pits are particularly important in degradation because they provide access for the invading mycelium to pass from one cell to another. When wood is dried, the pit membrane becomes aspirated or forced against one side of bordered pits, thereby blocking the free passage of water, oxygen, and possibly mycelia. Pit aspiration has been proposed as a reason for the slower degradation of gymnosperms by white-rot fungi, but investigations have not supported this idea (Highley, 1978). Pits are not essential for the passage of fungi such as white- and brown-rotters which can produce bore holes perpendicular to the cell axis. Hubert (1924) suggested that this ability could distinguish between such decay fungi and various staining fungi which merely inhabit the wood. Both white-rot and brown-rot degradative fungi initially invade through the pits, whereas in later stages of decay they produce bore holes.

White-rot and brown-rot fungi are commonly indicated and studied for their abilities to decay whole wood. White-rot fungi are generally associated with decay of angiosperms and brown-rot fungi with the decay of gymnosperms (Cowling, 1961) with white-rot fungi showing the more pronounced host selectivity (Eslyn and Highley, 1916). White-rot fungi degrade cellulose and hemicellulose more or less at the same rates while lignin is degraded at the same rate or slightly faster. By comparison, brown-rot fungi utilize the cellulose and hemicellulose leaving the lignin demethylated, but otherwise largely undigested (Highley and Kirk, 1979). In both instances, the hyphae are initially concentrated in the ray cells and propagate into the center of the wood along the inside of the cell lumens. Degradation of the cell occurs from the inside out. The mycelia of both white-rot and brown-rot fungi are surrounded by a hyphal sheath (Palmer *et al.*, 1983a,b) which, in the case of brown-rot fungi, spreads away from the fungus and sometimes encompasses the cellulosic fibrils (Highley *et al.*, 1983a; Highley *et al.*, 1983b). It is not clear whether the sheath of white-rot fungi is involved in cellulose degradation, but both white-rot and brown-rot fungi produce a diffusible cellulose-degrading system because degradation occurs at some distance from the mycelia (Highley and Murmanis, 1984).

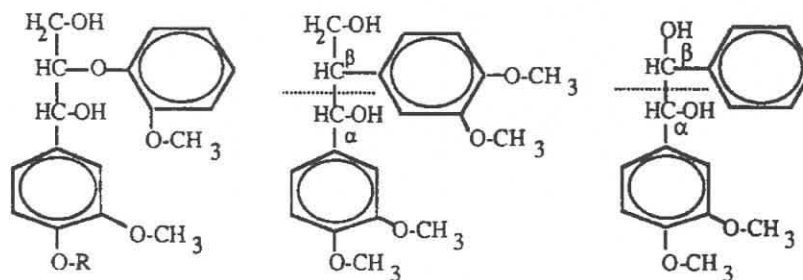
BIODEGRADATION OF LIGNIN

Biodegradation of lignin has been studied for more than 80 years with only incremental progress over much of the time (Crawford and Crawford, 1984). Early assays employed the disappearance of acid-insoluble Klason lignin. The development of synthetic ¹⁴C-labeled lignins (Kirk *et al.*, 1975) enabled rapid radiorespirometric determination of lignin degradation under controlled conditions, and a series of experiments using this assay led to improved understanding of the cultural and nutritional conditions under which lignin biodegradation occurs.

In *Phanerochaete chrysosporium*, a co-metabolizable carbon source is essential for lignin biodegradation (Kirk *et al.*, 1976), and it occurs in response to nitrogen starvation (Keyser *et al.*, 1978). This indicates that the ligninolytic system is formed as a part of secondary metabolism in this organism. Carbohydrate starvation likewise leads to a rapid but transient onset of ligninolytic activity (Jeffries *et al.*, 1981). Elevated oxygen levels increase the rate of lignin biodegradation (Kirk *et al.*, 1978), through the production of hydrogen peroxide as an extracellular oxidant and the induction of ligninolytic activity (Faison and Kirk, 1983). The hydrogen peroxide is derived from the cometabolism of cellulose and/or hemicellulose by reactions which are as yet incompletely characterized.

The complexity of lignin prevents meaningful biochemical studies on the polymer itself; so concepts about biodegradative mechanisms are derived from studies with biodegradation or modification of model compounds and direct chemical characterization of products from wood decayed under ligninolytic conditions. It is not completely clear whether the reactions observed in oligomeric substrates are completely related to what occurs in the native lignin polymer. For example, cleavage of aromatic rings in monomeric aromatic compounds was demonstrated several years ago using cellular extracts of a lignin-degrading fungus (Buswell and Erickson, 1979), but degradation of aromatic rings of lignin is now known to occur in the polymer (Haider *et al.*, 1985). Some of the earliest studies on lignin models were done on dimeric compounds containing the DL-glycerol β -aryl ether linkage (Russell *et al.*, 1961), the choice being based on the fact that this linkage accounts for most of the interunit bonds in lignin (Adler, 1977).

One of the principal problems in employing model compounds in whole cultures is to carry out the cleavage of interest while preventing subsequent reactions which lead to degradation or polymerization. For example, guaicyl-glycerol- β -guaicyl ether (I) contains a phenolic hydroxy group and is readily degraded by white-rot fungi containing phenol oxidase while the non-phenolic veratryl glycerol- β -guaicyl ether (II) is not degraded (Kirk *et al.*, 1968). The problem of sequential reactions is particularly acute because the initial reaction of interest is often rate-limiting. A major breakthrough in the design of model lignin substrates came with the use of the non-phenolic model compound 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol (III). Biodegradation of III in ligninolytic cultures shows that primary cleavage occurs between C_α and C_β with incorporation of molecular oxygen (Nakatsubo *et al.*, 1982). A recent review of how model compounds have elucidated the degradative pathway for lignin has been given by Higuchi (1985b).



Enzymes have now been identified in the cell-free extracellular broth of ligninolytic cultures which will depolymerize spruce lignin and catalyze the degradation of Lignin-related models (Tien and Kirk, 1983). Depolymerization is carried out by a 42 kd ligninase with a pI of 3.5 which requires hydrogen peroxide for activity. Ligninase was purified to homogeneity (Tien and Kirk, 1984) and shown to contain a single protoheme IX prosthetic group. Ligninase is similar to classical peroxidases in its resonance Raman spectra of the heme active site (Kuila *et al.*, 1985), and like peroxidase, H_2O_2 reacts stoichiometrically with the heme to give an oxidized intermediate. Following reduction, the enzyme reacts with the lignin model compound (Tien *et al.*, 1986) by abstracting an electron from the aromatic ring thereby leaving a cation radical (Kersten *et al.*, 1985). Once the cation radical is formed, C_α cleavage occurs spontaneously (Hammel *et al.*, 1985). A number of subsequent reactions, such as demethoxylation or C_α - C_β cleavage, can follow - largely dependent on the nature of the substrate.

Subsequent to the enzymatic reaction, the radicals can undergo other spontaneous reactions with the incorporation of oxygen. These sequential reactions complicate interpretation of the stoichiometries, and it is necessary to employ model substrates which exhibit only the reaction

of interest. One such compound is 1-(3,4-dimethoxyphenyl)-2-phenylethanediol (IV). Using this substrate, it was shown that substrate free radicals are formed as intermediate products (Hammel *et al.*, 1985). When acting on 1,4-dimethoxybenzene ligninase abstracts one electron at a time from the ring to produce cation radical intermediates which decompose through the addition of water and the removal of methanol groups to form *p*-benzoquinone (Kersten *et al.*, 1985). In recent studies of lignin model compounds representing the predominant arylglycerol- β -aryl ether substructure of lignin, Kirk *et al.* (1986a) showed that the major consequence of the ligninase activity is cleavage of the C α -C β linkage. C α oxidation is much less frequent and removal of aromatic alkyl ethers is a relatively minor reaction, although both types of reactions occur.

Other enzymes produced by *Phanerochaete* in liquid culture may play separate roles in biodegradation of lignin. For example, Huynh and Crawford (1985) have identified an extracellular H₂O₂-dependent aromatic methyl ether demethylase and an extracellular Mn⁺⁺ dependent peroxidase which has also been observed by Glenn and Gold (1985). At least 13 different proteins are found in the extracellular broth of *Phanerochaete* cultures, 10 of which appear to be haemoproteins (Kirk *et al.*, 1986b). When one also considers the possible roles of cellobiose:quinone oxidoreductase (Westermarck and Eriksson, 1974; Buswell, Hamp and Eriksson, 1979) and phenol oxidase (Ander and Eriksson, 1976) - if not in the initial depolymerization reactions then perhaps in subsequent reactions to drive the depolymerization reactions forward - then it is clear that the biochemical explanation of lignin degradation is far from complete.

Direct chemical studies of lignin degradation products in decayed wood do not always point to the same mechanisms or pathways as are revealed by model compound studies. Notably, a more prominent role for ring cleavage is indicated than is observed with model compounds (Chen and Chang, 1985). It is important to remember that lignin exists in wood in quite a different environment from model compounds in defined culture media. The localized concentration of substrate is very much higher and the availability of water is very much lower in wood than it is in culture broths. Also, lignin is found closely associated with hemicellulosic materials, the degradation of which could almost certainly affect lignin depolymerization. The differences, however, might simply reflect substrate accessibility. In the polymer, the aromatic rings would be attacked with the formation of cation radicals. The radicals would migrate to cleave the relatively reactive C α - C β linkages near the surface of the polymer. Those on the interior would be shielded from attack. The surface ring structures would subsequently be degraded by sequential electron abstraction.

Other aspects of lignin biodegradation such as the roles of oxidative species (Kirk *et al.*, 1984), details of biochemical mechanisms (Kirk, 1986), regulation (Kirk and Fenn, 1982) and bacterial lignin degradation (Crawford and Crawford, 1984) have been reviewed elsewhere.

BIODEGRADATION OF CELLULOSE

The biodegradation of cellulose has probably attracted more attention world-wide than any other single biochemical event outside the field of medicine, yet the mechanism of cellulose hydrolysis is still imperfectly understood. The cellulase complex from *Trichoderma reesei* has been studied most thoroughly, but it is not yet clear exactly how this complex works to depolymerize cellulose. The focus here is placed on the biochemical mechanisms involved in hydrolysis.

Reese *et al.* (1950) first postulated the C1 Cx hypothesis for cellulose hydrolysis to account for differences observed with crystalline and non-crystalline cellulose substrates. Since that time, the number of proteins known to catalyze cellulose depolymerization have proliferated, and they are now commonly classified into three groups (Table 3). Each of these is found in various isozymes, and their action on cellulose is synergistic. The cellobiohydrolases act on straight chain, unsubstituted cellulose and exhibit activity against crystalline cellulose. Endoglucanases will act on acid swollen straight-chain cellulose and on substituted cellulose such as carboxymethyl cellulose. β -glucosidases act on cellobiose and

aryl β -glucosides and their activity decreases rapidly as the degree of polymerization (DP) increases.

Table 3. Enzymes of the cellulase complex.

Systematic name	Enzyme no.	Trivial names	Substrate and product
1,4- β -D-glucan cellobiohydrolase	(EC 3.2.1.91)	<i>exoglucanase</i> , cellobiohydrolase	crystalline cellulose cellobiose
<i>endo</i> -1,4- β -D-glucan 4-glucanohydrolase	(EC 3.2.1.4)	<i>endoglucanase</i> , β -glucanase	amorphous cellulose cellooligosaccharides
β -D-glucoside glucohydrolase	(EC 3.2.1.21)	cellobiase, β -glucosidase	cellobiose, triose glucose

As is shown in Table 4, the principal endoglucanase (EG I) of *T. reesei* is very active against carboxymethyl cellulose (CMC) and shows essentially the same specific activity against phosphoric acid swollen cellulose (PSC). In contrast, cellobiohydrolase I (CBH I) shows more than 60 times as much activity against PSC than with CMC. Both CBH I and EG I show activity against avicel, but whereas CBH I can degrade it completely, EG I will attack it only until the amorphous regions are consumed. Only the EG I will attack xylan and only β -glucosidase (β G I) will attack cellobiose or *p*-nitrophenyl glucoside (PNPG) (Shoemaker *et al.*, 1983).

Table 4. Specific activities of cellulases isolated from *T. reesei* (Shoemaker *et al.*, 1983).

	Specific activity (U mg ⁻¹)					
	<u>Avicel</u>	<u>PSC</u>	<u>CMC</u>	<u>Cellobiose</u>	<u>PNPG</u>	<u>Xylan</u>
CBH I	0.04	0.6	<0.01	<0.01	<0.01	<0.01
EG I	0.17	26.0	27	<0.01	<0.01	6.4
β G I	<0.01	<0.01	<0.01	31.4	19.5	<0.01

Reese (1974) identified five criteria by which cellulases could be distinguished. Each gives some insight into the hydrolytic mechanism (Table 5). The first of these, effect of DP, has already been discussed. The second, retention or inversion of anomeric configuration is directly related to the hydrolytic mechanism. The mechanism for glycolysis has been characterized best by x-ray crystallographic studies of lysozyme.

Table 5. Classification criteria for cellulases (Reese, 1974).

<u>Criterion</u>	<u>Observation</u>
Effect of degree of polymerization	<i>em</i> have higher affinity on longer chain
Retention or inversion	<i>endo</i> retain; <i>exo</i> invert
Ability to transfer glycosyl	β -glucosidases are more able
Effect of lactone inhibitor	<i>endo</i> are more susceptible
Bond specificity	<i>exo</i> is more specific in its hydrolysis

Based on similar sequence homologies, it has been proposed that the catalytic sites of endoglucanases I and II (EG I, II), the cellobiohydrolase I (CBH I) and egg white lysozyme are similar (Paice *et al.*, 1984). Catalysis proceeds by an ion-pair mechanism. During binding, the C6 carbon of the glycone interacts with the enzyme to distort the glycone ring into a flattened half-chair. Catalysis is initiated by protonation from an acidic amino acid to the glycosidic oxygen. This brings about cleavage and leaves a positively-charged carbonium ion at the C1 of the glycone which is stabilized by ionic interaction with a negatively-charged amino acid residue on the opposite side of the ring. The glycone is finally released when the negatively-charged amino acid is displaced by a hydroxyl (Phillips, 1966; Eveleigh and Perlin, 1969). One feature of this mechanism is that it requires the substrate to sit in a groove in the enzyme, hence it might not be appropriate to the hydrolysis of a crystallite.

The anomeric configuration of the product depends on whether an enzyme-bound intermediate is formed. The presence of a bound intermediate is indicated by the ability of some glucanases to carry out transglycosylation reactions. Transglycosylation is most commonly seen with β -glucosidases but is also observed in endoglucanases to a lesser extent (Okada and Kisizawa, 1975). Cellobiohydrolases do not generally exhibit transglycosylation (Patil and Sadana, 1984; Reese, 1974). This observation combined with the fact that cellobiohydrolase does not retain the configuration of the C1 hydroxyl, indicates that no stable enzyme-bound intermediate is formed and anomeric inversion suggests that the hydroxyl displaces the glycosidic oxygen in a single step mechanism.

Endoglucanases do not exhibit a great deal of specificity with regard to the position of the linkage hydrolyzed, but they are specific with regard to the aglycone portion of the binding site, as indicated by the products formed through action on mixed-linkage glycans (Parrish and Perlin, 1960). Cellobiohydrolases are not active on mixed linkage β -glucans nor do they hydrolyze hydroxyl-substituted celluloses such as CMC.

The presence of the half-chair intermediate in hydrolysis by endoglucanases is indicated by the inhibitory activity of substrate analogs. One of the forms of D-glucono-1,5-lactone is very similar to the half-chair intermediate D-gluconopyranosyl cation formed during hydrolysis. D-glucono-(1,5)-lactone is a potent inhibitor of endoglucanases but is much less effective with cellobiohydrolase (Reese *et al.*, 1971). The glycolytic mechanism for *exo*-splitting hydrolases such as cellobiohydrolase is almost certainly different from that of *endo*-splitting hydrolases. Probably the cellobiose structural repeat unit of cellulose accounts for its appearance as a product.

In recent years, cellobiohydrolases of several fungi have been separated and characterized (Mishra *et al.*, 1983; Beldman *et al.*, 1985; Shoemaker *et al.*, 1983; van Tilbeurgh *et al.*, 1985) as two immunologically distinct proteins (Fagerstam and Pettersson, 1979) that show no sequence homology (Fagerstam and Pettersson, 1984). Amino acid sequence homology does, however, exist between CBH I and EG I (Shoemaker *et al.*, 1983; Paice *et al.*, 1984). CBH I and II are similar in that both show high affinity for crystalline cellulose and release cellobiose as their principal product. But CBH II shows greater specific affinity for cellobiose than CBH I (van Tilbeurgh *et al.*, 1984). Surprisingly, cellobiohydrolases show little specificity for the 4-OH non-reducing terminal substituent, and they can be differentiated by their relative abilities to bind and cleave a series of methyl umbelliferol (4-O-MeUmb) substituted cellooligosaccharides. CBH I will hydrolyze 4-MeUmb-(Glu), 4-MeUmb-(Glu)₂ or 4-MeUmb-(Glu)₃. CBH II in contrast will not hydrolyze any model smaller than 4-MeUmb-(Glu)₃, and even then its specific rate is 10-fold lower than that of CBH I on this substrate. Based on these results, van Tilbeurgh *et al.* (1985) have proposed that CBH I and II are able to interact with different cellobiosyl groups within longer chains. This finding also supports a proposed mechanism for the synergism observed between these two enzymes.

Structural asymmetry was first proposed by Fagerstam and Pettersson, (1980) as the basis for synergism between CBH I and CBH II. This notion has recently been given a more definite form by Wood *et al.*, (1986). When crystalline cellulose is used as a substrate, synergism between CBH I and CBH II is observed. However, when acid swollen cellulose is employed, synergism is negligible. Wood *et al.* (1986) have speculated that these two

different types of enzymes might attack two different types of non-reducing end groups which should arise in the cellulose crystallite (Fig. 3). This sort of synergism is different from that which has been long recognized between *endo*- and *exo*glucanases and between cellobiohydrolase and β -glucosidase (Wood and McCrae, 1979; Kanda *et al.*, 1980; Henrissat *et al.*, 1985). The synergisms are related to the creation of new enzyme cleavage sites and the relief of feedback inhibition.

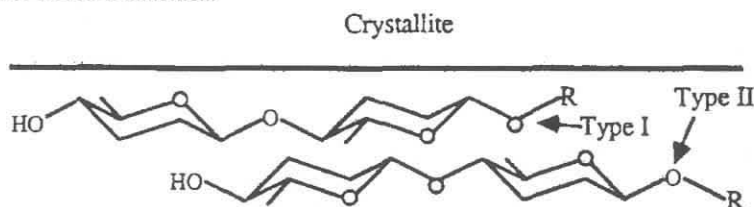


Fig. 3. Model of asymmetric glycosidic linkages in cellulose crystallite.

Purified CBH I is capable of attacking and degrading microcrystalline cellulose from *Valonia* without any other enzyme present (Chanzy *et al.*, 1983). When the enzyme is labeled with colloidal gold, its attachment can be visualized. The enzyme binds to the edges rather than the face of the crystallite. (Chanzy *et al.*, 1984). The specificity of CBH I is shown strikingly in its unidirectional attack on the non-reducing end of the molecule, giving rise to a tapered point (Chanzy and Henrissat, 1985). The reducing end is left untouched, as are the kinks in the crystallite (Fig. 4). If, however, a purified endoglucanase is added, hydrolysis is initiated at the kinks and multiple unidirectional hydrolysis is observed.

When all of the cellulases are present, *Valonia* crystals undergo a delamination to form bundles of microcrystallite fibrils (Chanzy and Henrissat, 1983). A similar delamination has been observed with the purified bacterial cellulose from *Acerobacter xylinum* (White and Brown, 1981). Although no direct evidence is available, the delamination probably occurs between the sheets adjacent van der Waals-bonded sheets rather than between adjacent hydrogen bonded chains.

The distinction between CBH I and II is not confined to *T. reesei*. CBH I and II are also found in the cellulase complex of *Penicillium pinophilum* (Wood *et al.*, 1986) and in *Fusarium lini* (Mishra *et al.*, 1983). In the latter instance, CBH I was found to be active against D-xylan, whereas CBH II was not. Given the apparent importance of the C6 carbon in forcing the half-chair conformation during the hydrolysis of the glycosidic bond by lysozyme (Phillips, 1966), it is apparent that substrates lacking this substituent may not be appropriate for some cellulases.

Reese (1974) pointed out that most cellulases don't act on xylan. This was puzzling in view of the fact that xylan did not possess additional side groups which might cause steric hinderance. It is possible that they use a mechanism similar to lysozyme to bend the ring into a reactive conformation. It is now apparent that in some instances at least, highly purified cellulase preparations possess activity against xylan (Shoemaker *et al.*, 1983), albeit at an insignificant rate in comparison to a true xylanase.

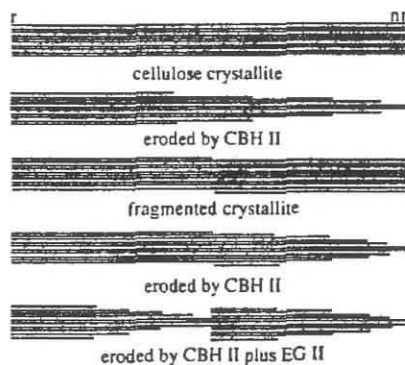


Fig. 4. Degradation of crystalline cellulose by purified cellulases.

BIODEGRADATION OF HEMICELLULOSE

Because of the diversity of hemicelluloses, it is not possible to survey here all of the different types known. Dekker and Richards (1976) have provided an extensive, useful compilation of this sort. Likewise, Reilly (1981), Woodward (1984) and Biely (1985) have recently reviewed the literature on microbial xylanases. Mannanases have been studied to a lesser extent, hence, the emphasis here is placed on xylanases.

The characterization of hemicellulase mechanisms presents numerous difficulties not encountered with cellulase because the substrate employed is heterogeneous and branched. Moreover, the literature is not definitive because substrates such as larch arabinoglucuronoxylan commonly used for hemicellulose assays –presumably because of their easy solubility – but they are not really representative of the bulk of hemicellulose found in wood. Selection of an appropriate substrate is critical because the apparent specific activity of a xylanase can vary greatly depending on the xylan preparation used for assay. Substrate variability is not confined to the type of xylan employed, because different batches of xylan from the same supplier can lead to apparent changes of as much as two-fold (Khan *et al.*, 1986). Native xyans tend to be highly acetylated. Acetyl groups increase solubility but decrease the rate of enzymatic depolymerization (Biely *et al.*, in press); so the method of substrate preparation can have a profound affect on the apparent activity.

Xylanases, like cellulases, can be classified into β -xylosidases, *exo*- β -xylanases, and *endo*- β -xylanases (Reilly, 1981; Frederick *et al.*, 1985). The endoxylanases can be further differentiated on the basis of their abilities to cleave at L-arabinose branch points or produce xylooligosaccharides. Like β -glucosidases, β -xylosidases don't carry out inversion of the C1 anomeric configuration and they exhibit strong transferase activity indicating the presence of a stable enzyme-bound intermediate. β -Xylosidases are strongly inhibited by their end product, xylose. *Exo*- β -xylanases, like *exo*- β -glucanases invert the anomeric configuration of the C1 hydroxyl during hydrolysis and do not exhibit transferase activity. Most do not show strong endproduct inhibition, but they are commonly inhibited by divalent cations, particularly Hg^{++} (Matsuo *et al.*, 1977; Takahashi and Kutsumi, 1979), indicating that a free sulfhydryl group might be involved in the catalytic mechanism. Endoxylanases cleave throughout the xylan polymer leaving xylobiosyl and xylotriosyl residues attached to L-arabinofuranose (Takenishi and Tsujisaka, 1973). The substrate specificities and hydrolytic products of endoxylanases have been best characterized in a series of enzymes purified from *Aspergillus niger* (Frederick *et al.*, 1981; Frederick *et al.*, 1985; Shei *et al.*, 1985; Fournier *et al.*, 1985). Some appear to require a side branch for maximal attack, but this may simply be related to solubility or accessibility of the substrate.

Biochemically, *endo*- β -(1,4)-D-xylanases can possess much higher turnover numbers than can equivalent *endo*- β -(1,4)-D-glucanases. For example, the V_{max} of an endoxylanase from *Trichoderma harzianum* was found to be 580 U mg^{-1} of protein (Tan *et al.*, 1985), or more than 20 times the equivalent rate observed with the major endoglucanase of *T. reesei* (Table 5). As was pointed out earlier, this difference in rate is attributable at least in part to the relative instability of the β -(1,4) glycosidic bond in xylose.

Xylotriose and xylopentaose have been observed as products of endoxylanases having specificity for long-chain, unsubstituted xylan (Fournier *et al.*, 1985). The presence of xylotriose as a predominant product could be related to the xylotriosyl repeat unit observed in x-ray crystallographic studies of xylan (Marchessault and Liang, 1962). The possible involvement of a DP 5 oligosaccharide in binding is reinforced by the observation that xylopentaose is a strong competitive inhibitor of xylanase from *Trichoderma viride* (Toda *et al.*, 1971). These results can be understood if xylanases having preference for straight-chain, unsubstituted xylan preferentially cleave off a xylotriose repeat unit but will not hydrolyze an oligosaccharide with a DP <8.

Many xylanases are not able to remove glucuronic acid residues from the xylan chain (Biely, 1985) probably because of the 30-fold greater stability of glucuronoside glycolytic

linkages noted earlier (Harris, 1975). By comparison, the ester linkages connecting acetyl groups to the xylan are exceedingly unstable and most are removed during isolation of the polymer. Although synergism is observed between xylan esterases and endoxylanases during hydrolysis of partially acetylated xylan, (Biely *et al.*, 1985; Biely *et al.*, in press), it seems unlikely that deacetylation would be a rate-limiting step in the enzymatic hydrolysis of xylan under most practical conditions because of the extreme lability of the ester linkages.

APPLICATIONS

Technical developments, particularly the discoveries of xylose-fermenting yeasts (Jeffries, 1985) and lignin-degrading enzymes have paved the way for specific removal of hemicellulose and lignin in order to recover cellulosic fibers and to produce useful chemicals from the hemicellulose and lignin fractions.

Biological processes might be applied directly to the removal of lignin from whole wood chips by fungi. Lignin removal need not be complete in order to realize a benefit in reduced energy input during thermo-mechanical pulping (Eriksson and Vallanders, 1982). The major problem is in obtaining selective removal of lignin without concomitant cellulose degradation. One approach has been to incorporate glucose into the chips in order to repress cellulase production (Kirk and Yang, 1979; Bar-Lev *et al.*, 1982). Another approach has been to obtain cellulose deficient mutants which will still degrade lignin at the expense of hemicellulose (Eriksson, *et al.*, 1980). The cellulase negative mutants presently available show various ligninase activities (Kirk *et al.*, 1986b). Some native strains of fungi exhibit fairly selective removal of lignin (Blanchette, 1984a,b; Otjen and Blanchette, 1984). Such organisms are presently being evaluated for their abilities to remove lignin from chips. Other applications of lignin degrading fungi in biopulping, biobleaching and waste treatment in the pulping industry have been reviewed recently (Eriksson and Kirk, 1985).

An alternative approach to biological pulping which permits more direct control over the degradation activities is to employ isolated enzymes for lignin or hemicellulose removal. Because the lignin is cross-linked mostly into the hemicellulose, because the hemicellulose is accessible due to its porosity and because hemicellulose is more readily depolymerized than lignin, it has been the initial target of treatment (Paice and Juraš). A small portion of the hemicellulose might be sufficient to open up the polymer and ease solvent removal of the residual lignin. The principal problem in these studies has been in obtaining selective removal of hemicellulose without degrading the cellulose. Selective inhibition of cellulase activity can be realized by the addition of 1 mM HgCl_2 (Mora *et al.*, 1986). Xylan was specifically removed from delignified cell walls leading to a decrease in energy demand during beating (Noé *et al.*, 1986). Removing less than 2% of the initial dry pulp weight gave better fiber bonding due to increased flexibility (Barnoud *et al.*, 1986). Therefore, enzymatic treatments of pulp hold the prospect of both decreased costs and improved fiber qualities.

CONCLUSIONS

The physical structure of the tissue and the microstructures of the components determine the accessibility of degradative organisms and enzymes. During the degradation of whole wood, mycelial fungi invade by ray cells and move throughout the tissue by way of the intracellular lumens and pit connections. Degradation of the cells takes place from the inside out with hemicellulose being removed first. Lignified, highly cellulosic tissues are degraded later. The presence of coherent hydrogen bonding in cellulose and the tight covalent cross-linking in lignin prevent access of enzymes to the interiors of these molecules; so erosion occurs principally from the outside. The mechanisms for lignin degradation are determined by the relative chemical stabilities of the bonds, the Ca linkage being the weakest. Likewise, cellulose crystallites are more resistant than unbonded chains, so degradation proceeds first through removal of amorphous regions. Degradation of the cellulose crystallite proceeds through synergistic activities of several *endo*- and *exo*glucanases. Substitution of

hemicellulose, particularly by uronic acids inhibits degradation, but is open, hydrated structure is both accessible and relatively easy to depolymerize. As porosity increases, diffusion of oxygen and other enzymes into the wood cell structure increases. Removal of hemicellulose can improve the quality of pulps, improve access to and removal of lignin, and may lead to processes in which fuels, chemicals and fibers are co-produced from wood feedstocks.

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